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(54) Title: BACULOVIRUS EXPRESSION SYSTEM AND METHOD FOR HIGH THROUGHPUT EXPRESSION OF GENETIC MATERIAL

(57) Abstract

The present invention provides novel recombinant baculovirus expression systems for expressing foreign genetic material in a host cell. Such expression systems are readily adapted to an automated method for expression foreign genetic material in a high throughput manner. In other aspects, the present invention features a novel automated method for determining the function of foreign genetic material by transfecting the same into a host by way of the recombinant baculovirus expression systems according to the present invention.

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BACULOVIRUS EXPRESSION SYSTEM AND METHOD FOR HIGH THROUGHPUT EXPRESSION OF GENETIC MATERIAL

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FIELD OF THE INVENTION

The present invention relates generally to the field of molecular biology and genetics. Specifically, the present invention relates to novel baculovirus expression systems and methods for expressing foreign genetic material by tranfecting the same into insect cells.

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BACKGROUND OF THE INVENTION

A large amount of sequence information has been produced describing the genetic code of many smaller organisms. Tremendous accomplishments have been achieved in a far more expeditious manner than previously believed possible to similarly describe the genetic make-up of higher organisms such as humans. Undoubtedly, efforts will be intensified to accomplish sequencing the genetic code of these organisms, particularly those important to humans such as plants and other mammals. There now exists a particular need to develop rapid and accurate means for determining the functions of the particular genes and introns whose sequences are described by these sequence data in order to make use thereof. The first step in such an endeavor is expressing the gene products of sequenced genetic material. Critical technology involved in efficiently accomplishing this goal will likely utilize robotics capable of handling many gene or gene product samples simultaneously. Moreover, optimal methods for purifying expressed gene products will be utilized to further facilitate the process.

Baculoviruses infect insect cells. Both prokaryotic and eukaryotic cells have been employed to express foreign genetic material. However, prokaryotic cells such as *Eschericia coli* are suitable for expressing foreign genetic material only if the gene product does not require post-translational modification such as glycosylation, phosphorlyation or signal peptide cleavage. Prokaryotic cells do not contain the machinery needed for such post-translational modification. Hence, it is particularly important to develop novel and easily manageable recombinant expression systems in eukaryotic cells. The insect cells which baculoviruses infect accomplish most eukaryotic post-translational modifications (including phosphorylation, N- and O-linked glycosylation, acylation, disulphide cross-

linking, oligomeric assembly and subcellular targeting), and this has been exploited to produce functional recombinant protein from many diverse foreign genes.

Genetically modified baculoviruses are widely used to produce recombinant proteins. Davies, Bio/technology 12:47-50 (1994). Exemplary baculoviruses include but 5 are not limited to *Autographa californica*, *Trichoplusia ni*, *Rachiplusia ou*, *Galleria mellonella* and *Bombyx mori*. Of these, *Autographa californica* is probably the most fully characterized and widely used. The polyhedrin gene of baculoviruses is expressed at very high levels during the natural viral life cycle, but is dispensable in cell culture. This allows it to be replaced by almost any heterologous nucleotide sequence of interest. Any 10 heterologous nucleotide of interest inserted so as to replace the polyhedrin gene may itself be controlled by the polyhedrin promoter, and thus expressed to similarly high levels.

Manipulation of baculovirus genomes at the molecular level is challenging, as they comprise some 130 kb of DNA too large to be amenable to conventional plasmid cloning techniques. The traditional solution to this problem has been to introduce foreign genes by 15 homologous recombination, as a cassette also comprising suitable promoter and termination sequences. This is accomplished by flanking the cassette, in a plasmid vector, by the viral DNA sequences which flank the point at which it is to be inserted. The efficiency of this process is usually less than 1%.

In the past, plaque purification has been used to proceed from this point to a clonal 20 vector. Some traditional procedures are described by Smith *et al.*, U.S. Patent 4,745,051, Smith *et al.*, U.S. Patent 4,879,236, Summers *et al.*, U.S. Patent 5,169,784, Guarino *et al.*, U.S. Patent 5,077,214, Kang, U.S. Patent 5,194,376, Matsuura *et al.*, U.S. Patent 5,229,293 and Murphy *et al.*, U.S. Patent 5,516,657, the disclosures of which are herein incorporated by reference. Recently, however, several different techniques have been 25 developed to increase the frequency of recombination into the baculovirus genome. The most successful of these are described, *infra*.

The baculovirus genome has been reconstituted as a replicon which will propagate in the yeast *Saccharomyces cerevisiae*. This was achieved by inserting a yeast 30 Autonomous Replicating Sequence (ARS) into the polyhedrin locus of the baculovirus genome, along with a CEN (centromeric) sequence, which ensures stable low copy number segregation of the genome by acting as a mitotic centromere, and the URA3 selectable marker, to permit growth in a uracil-free medium. Thus the recombination of a polyhedrin

promoter-driven foreign gene may be undertaken in yeast, and the resulting baculovirus genome extracted from the yeast cells and transfected directly into insect cells as a clonal virus. Advantages of this method include near 100% efficiency and the ability to manipulate in a foreign host genes whose products might be toxic to insect cells. However, 5 a large number of manipulations are required making this procedure cumbersome and complicated.

The baculovirus genome has also been reconstituted as a replicon which may propagate in *Escherichia coli*. In a similar approach to that used in yeast, Luckow *et al.*, *J. Virology*, 67: 4566-4579, initially demonstrated that the baculovirus genome may be modified to replicate as a large plasmid in *Escherichia coli*, termed a 'bacmid'. This may be performed by recombining a mini-F replicon into the polyhedrin locus, conferring autonomous replication and stable, low-copy number segregation of the genome, and the kanr selectable marker. The target site for the Tn7 bacterial transposon may also be introduced, as an in-frame insertion within the lacZa sequence from a pUC-based plasmid. 10 This bacmid may therefore intra-allelically complement the defective β -galactosidase lacZDM15 of *Escherichia coli* hosts such as DH 1 OB. However, *Escherichia coli* DH 1 OB harboring a bacmid with a foreign gene inserted at this Tn7 site would remain lacZa-, enabling visual selection of colonies containing recombinant bacmids. The overall strategy 15 of such a procedure is to accomplish the recombination and selection steps in the heterologous host, here *Escherichia coli*, and only then to transfer the finished product to insect cells. This strategy has similar advantages to the yeast system but also involves many steps.

Rather than reconstitute the baculovirus replicon in a heterologous host considered preferable for selection of recombinant species, an *in vitro* recombination reaction to 20 transfer genes from transfer vectors to the polyhedrin locus of the virus has been developed. Exploiting the Cre recombinase of bacteriophage Pi and its substrate lovp, the gene transfer in this system is achieved by a single enzymatic crossover reaction. Both the target baculovirus genome (vaclox) and the transfer vector are engineered to contain *lox* sites. These 34 nucleotide sequences direct the Cre enzyme to convert the two substrate 25 DNA molecules into topologically unlinked, recombinant products. The reaction proceeds stoichiometrically, with an efficiency of around 70%. This approach has the great advantage of simplicity, however, the maximum efficiency is only about 70%.

In 1990, Kitts *et al.*, *Nucl. Acids Res.* 18:5667-5672 (1990) derivatised wild-type baculovirus DNA, which exists as a covalently closed circular double-stranded molecule, by introducing a unique restriction site (*Bsu36I*) at the polyhedrin locus. Linearising the baculovirus genome using this restriction site reduces the infectivity of the viral DNA on 5 transfection into insect cells, however, cotransfection with a transfer vector driving recombination into the polyhedrin locus produces a three fold higher proportion of recombinant viruses.

In this strategy, the baculovirus containing the *Bsu36I* site was designated ACRP-SC (for single cut), and the two double crossover events transferring the foreign gene to be 10 expressed, along with its own copy of the polyhedrin promoter and terminator sequences, are also present. About 10 to 25% of the progeny viruses from such a cotransfection are recombinant, but it should be noted that this is due to the reduced background of wild type viruses rather than an increase in the absolute number of recombinants.

A subsequent development of this system effectively combines it with both a visual 15 (*lacZ*-based) and a replication-based selection strategy. Kitts *et al.*, *Biotechniques* 14:810-817 (1993). Recombination occurs between a conventional transfer vector and a derivative of the wild type genome, containing *lacZ* at the polyhedrin locus and two further *Bsu36I* sites in the polyhedrin flanking sequences (designated BacPAK6). *Bsu36I* digestion of this baculovirus DNA not only linearizes the molecule, but also removes two genomic 20 fragments, thus disrupting the open reading frame ORF-1629. This gene is essential for baculovirus replication, so, with the two *Bsu36I* fragments removed from the genome, competent viruses will only be reconstituted by recombination with the transfer vector, whereby an intact ORF-1629 will be restored to the genome. Furthermore, recombinant viruses will form white plaques on a background of blue plaques from BacPAK6 DNA.

This strategy yields recombinant viruses at a frequency of 85-99%. The majority of 25 the background may be attributed to contamination with undigested BacPAK6 DNA, which will be substantially more infectious than linear forms of the genome. Viruses which offer the prospect of utility for future work, which are likely to be a small minority of the total number of viruses generated, may then be purified by conventional technology 30 (plaque assays). Therefore this method offers a good combination of simplicity and efficiency, and will be our technique of choice for converting arrayed cDNAs into expression vectors.

One particular problem associated with baculovirus expression systems is that they may produce apoptosis or cell lysis of infected cells. A baculovirus apoptosis resistance gene (p35 gene) has been identified that confers increased viral yield in certain cell lines. Recombinant baculoviruses bearing this gene can selectively amplified (up to 10^6 fold) in appropriate hosts.

Despite the useful combination of high yields and authenticity of processing associated with baculovirus expression systems, purification of the desired product from baculovirus infected cells is no easier than from any other eukaryotic system. Many expression vectors have been developed enabling synthesis of the antigen of interest as a fusion with a polypeptide facilitating purification. For instance, protein A fusion proteins may be affinity purified on IgG, polyarginine fusion proteins may be purified by cation exchange, polyhistidine fusions may be purified by virtue of their chelation of zinc ions, β -galactosidase fusions, and other fusions to specifically immunogenic partners, may be purified by immunoaffinity, and β -galactosidase, maltose binding protein and glutathione-S-transferase (GST) fusion proteins may be purified by substrate affinity. Other epitope tags such as those recognized by the EE or Glu-Glu antipeptide antibody may also be used. This antibody was raised against a peptide that comprises the major tyrosine phosphorylation site of polyoma middle t antigen. This tag has been used extensively in many recombinant applications. Some investigators report that over 90% of attempts to purify EE-tagged proteins have been successful, most of these to over 50% purity with no other chromatographic steps (Jim Litts and Robin Clark unpublished data).

The epitope for this tag has been fully characterized and optimized by NEmotope peptide scan analysis (Mario Geysen, John Wang and Robin Clark unpublished data). The antibody has a moderate affinity for the EE tag ($K_d - 2 \times 10^7$) which allows rapid elution of tagged proteins by free peptide under non-denaturing conditions while retaining efficient binding of proteins in crude lysates. While usually placed at the N-terminus, the EE tag is also recognized when placed at the C-terminus or at internal portions of the protein. An antibody to the tag is also useful for immunoprecipitates, immunofluorescence and western blots. Moreover, the antibody is produced at high levels by the EE hybridoma. Some have reported that 10 L preps routinely yield 3-5g of purified antibody. The EE tag has the additional advantage of containing a strong tyrosine phosphorylation substrate for protein kinases such as src. Such a tag may be directly labeled with radioactive phosphate or detected with an anti-phosphotyrosine antibody.

SUMMARY OF THE INVENTION

In one aspect, the present invention features novel baculovirus expression systems especially adept at expressing heterologous genetic material in a host. Generally, such systems comprise one or more selectable marker genes, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites. Exemplary selectable marker genes include an apoptosis suppressor gene such as AcNPV p35 which may be located upstream from a 5 polyhedrin promoter in many embodiments. Exemplary tags include the EE tag sequence.

10 In preferred embodiments, the ORF-1629 gene is also present in the baculovirus expression system, and is used as a second selectable marker gene. The invention features a method for producing recombinant baculovirus in which a DNA fragment containing foreign genetic material is used to combine preferably two selectable marker genes into a 15 single DNA fragment which, when co-transfected with Baculovirus DNA into insect host cells, yields a recombinant baculovirus which can be isolated by selection of selectable marker genes. Therefore, the present invention can be used to produce recombinant baculovirus without utilizing cloning steps, and thereby is useful for automated high throughput expression systems.

20 In a second aspect, the present invention features a method for producing a recombinant baculovirus expression system comprising the steps of (a) providing a baculovirus transfer vector comprising an apoptosis suppressor gene, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites, and (b) ligating 25 foreign genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter.

30 In a third aspect, the present invention features a method for expressing foreign genetic material comprising the steps of: (a) providing a baculovirus transfer vector comprising one or more selectable marker genes, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment

thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites, (b) ligating foreign genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter, and (c) transfecting the baculovirus transfer vector comprising the foreign genetic material into a host cell. In preferred embodiments, the present invention is adapted to automation and thereby is useful for high throughput expression.

In a fourth aspect, the present invention features a method for determining the function of foreign genetic material comprising the steps of: (a) providing a baculovirus transfer vector comprising one or more selectable marker genes, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites, (b) ligating foreign genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter, (c) transfecting the baculovirus transfer vector comprising the foreign genetic material into a host cell, and (d) observing the biological results produced thereby. In preferred embodiments, the present invention is adapted to automation and thereby is useful for high throughput analysis.

In a fifth aspect, the present invention features genes and fragments thereof, nucleotide sequences, and gene products obtained by way of the method of the present invention. The present invention features expressing selected nucleotide sequences in a host organism. Those of skill in the art will readily appreciate that the gene products of such nucleotide sequences may be isolated and purified using techniques known to those skilled in the art. Such isolation and purification techniques may be optimized according to some of the methods described herein. Such gene products may exhibit biological activity as pharmaceuticals and other similar functions.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 demonstrates a general schemata for a high throughput protein function assay which may be performed according to the methods of the present invention. Selected cDNA clones may be amplified, optionally tagged and transfected into recombinant baculovirus transfer vector which have been linearized in an arrayed configuration. The recombinant baculovirus expression systems may in turn be transfected into suitable host cells such as insect cells. The product of the foreign genetic material may

be purified by any suitable purification protocol such as affinity means. Optionally, the purified proteins may then be further analyzed in order to determine the biological activity and/or function thereof.

FIGURE 2 demonstrates the applicability of the present invention to expressing genetic material such as EST cDNA clones. A baculovirus comprising a p35 apoptosis suppressor gene, a polyhedrin promoter, an EE tag, and a cloning site under the control of the polyhedrin promoter may be ligated to foreign genetic material such as EST cDNA clones such that the foreign genetic material is expressed at high levels. The baculovirus expression system containing the foreign genetic material may then be transfected into host cells such as insect cells to effect expression of the product of the genetic material. EST cDNA clones may be amplified according to PCR procedures well known in the art and suitably prepared for cloning into the cloning site of interest. Such a procedure results in high transcription and high translation rates of the foreign genetic material thereby allowing for high level protein production and enabling rapid analysis of gene functions.

FIGURE 3 demonstrates enzymatic detection of recombinant proteins in a 96-well plate format using a recombinant baculovirus expressing β -glucuronidase (Gus). Sf9 cells grown in insect serum free medium were grown to mid-log phase and used to seed 96-well flat bottom polystyrene tissue culture plates at a density of 40,000 cells/100 μ l/well. rGus virus was serially diluted in 10-fold increments. After addition of virus dilutions, the plate was incubated for several days, and then X-glucuronide stock was added to each well. X-glucuronide is a histochemical substrate for β -glucuronidase, yielding a blue substrate upon hydrolysis. Blue color development was visible in many wells within minutes after addition of the substrate, and color development continued to occur in high dilution wells over 24 hours as rGus production increased in infections resulting from low MOI's.

FIGURE 4 demonstrates the results obtained when Millipore MAIP N45 high protein binding plates containing Immobilon-P filtration membranes were treated with either Glu-tagged or GST-tagged proteins and assayed for binding using a monoclonal anti-Glu Glu antibody and an alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody. Alkaline phosphatase was detected using SigmaFast BCIP/NBT tablets. These data demonstrate that purified Rho-EE, E2F-EE and Mut1-EE were detected at concentrations ranging from 0.1 to 2.0 μ g/well.

FIGURE 5 shows that the scanned image of a 96-well plate shows that recombinant baculoviruses can be efficiently produced without any cloning steps using the invention methods. A marker gene (GUS) whose expression can be detected colorimetrically was cloned into one of the IMAGE library vectors (Stratagene bluescript). Bacterial cultures of 5 this clone were grown in a 96-well plates and used for PCR (directly from bacteria). PCR primers were designed to hybridize to vector sequences and contain sequences for Ligation Independent Cloning (LIC). The resulting products were digested with T4 DNA polymerase, annealed, and ligated to recombination arms (also prepared for LIC by with T4 DNA polymerase). The ligation products were then co-tranfected with linearized baculovirus 10 (polyBSU2 cut with Bsu36I) into Sf9 cells seeded into a 96-well plate. The resulting virus was passaged 3 times on Sf9 cells to amplify and normalize viral titers. Cells from the final passage were stained with Magenta-gluc (a colorimetric substrate for GUS) to detect the presence of recombinant baculovirus (indicated by magenta color). The image shows that 78 of 88 experimental wells contain recombinant virus.

15 DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention features novel baculovirus expression systems especially adept at expressing heterologous genetic material in an insect host. Generally, such systems comprise one or more selectable marker genes, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription 20 termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites. Exemplary selectable marker genes include AcNPV p35 which may be located upstream from a polyhedrin promoter in many embodiments and which may be located downstream from 25 the transcription termination sequence in many embodiments. Exemplary tags include the EE tag sequence and preferably the ORF-1629 gene.

In some embodiments, the present invention features recombinant baculoviruses that are generated by transfecting insect cells with ligated fragments containing transfer vector elements, selectable markers and cDNA as opposed to cloned transfer vector 30 plasmids as taught by the prior art. Therefore, the baculovirus expression systems of the present invention greatly simplify the methods of the prior art by eliminating the plasmid cloning steps and by allowing the expression of cDNA which cannot be cloned in bacteria. In this embodiment, selectable markers residing on separate fragments may be jointed to

fragments containing a cDNA coding sequence. This provides for the selection of recombinant baculoviruses which contain the desired cDNA. Any method suitable for joining DNA fragments may be applied for this purpose.

The recombinant baculovirus expression systems of the present invention comprise
5 in the most preferred embodiments an apoptosis suppressor gene encoding the p35 protein, a polyhedrin promoter, foreign genetic material under the control of the polyhedrin promoter and cloned into the baculovirus at cloning sites, a tag sequence such as the EE tag either upstream or downstream from the cloning site and a ORF-1629 gene which is believed to be important for baculovirus replication and/or infectivity. It is further
10 contemplated that convenient restriction sites will be found both upstream and downstream from all of these elements though they need not be adjacent to any one particular element. Such convenient restriction sites include but are not limited to a Xhol site and a PvuII site

In a second aspect, the present invention features a method for producing a recombinant baculovirus expression system comprising the steps of (a) providing a
15 baculovirus transfer vector comprising one or more selectable markers, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites, and (b) ligating
20 foreign genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter.

In some embodiments such a recombinant baculovirus expression system may be produced in an automated or high throughput fashion. It is specifically contemplated that an operable portion of a baculovirus comprising an apoptosis suppressor gene, a promoter
25 such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag may be removed from the baculovirus genome using restriction enzymes.

30 In such embodiments of the present invention wherein recombinant baculovirus expression systems are produced using automation, high yields may be produced in a relatively short period of time. Using such technology, such genetic material as cDNA

may be arrayed for insertion at a cloning site so that recombinant baculoviruses containing many differing inserts may be provided.

In a third aspect, the present invention features a method for expressing foreign genetic material comprising the steps of: (a) providing a baculovirus transfer vector comprising an apoptosis suppressor gene, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites, (b) ligating foreign genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter, and (c) transfecting the baculovirus comprising the foreign genetic material into a host cell.

It is generally contemplated that in preferred embodiments the host cell is an insect cell. Baculoviruses have been shown to be pathogenic to some species of insect cells.

Thus, in preferred embodiments, an apoptosis suppressor gene is provided in the recombinant baculovirus expression systems of the present invention. Sf9 has been specifically demonstrated as a particularly promising animal cell host for the recombinant production of proteins according to some embodiments of the present method.

It is specifically contemplated that the cloning of foreign genetic material and isolation and purification of the products obtained from the expression thereof may be automated. The technologies allowing parallel processing of samples are a combination of organizational formats, particularly the widespread use of 96 and 384 well microtiter plates and the increasing use of sample handling robots designed to rapidly and systematically work with such organizational formats. For instance, the 96 well round microtiter plate was initially developed specifically for use in the field of immunology, but the 384 well was developed by Lawrence Livermore National Laboratory for use in genome research. Moreover, early robots have been designed for use with the 384 plates to facilitate genomic research. Copeland *et al.*, *Nature* 369:421-422 (1994).

Concomitant with the formatting and automation developments, cloned samples of genomic DNA and complementary DNA, hereinafter referred to as cDNA, have increasingly been provided in such formatted arrays. For instance, cosmid and phage libraries containing inserts primarily from single human chromosomes isolated by flow-sorting were provided by Lawrence Livermore National Laboratories and Los Alamos

National Laboratory as part of the Department of Energy's National Gene Library Project. Recently, cDNA clones have become available in large numbers through the I.M.A.G.E. (Integrated Molecular Analysis of Genomes and their Expression) Consortium at Lawrence Livermore National Laboratories. Lennon *et al.*, *Genomics* 33:151-152 (1996).

5 The I.M.A.G.E. Consortium promotes the use of shared and arrayed cDNA libraries. In partnership with Washington University (Hillier *et al.*, *Genome Research* 6:829-845 (1996)), sequences from these publicly available clones are deposited without restriction or delay in the public sequence databases, a database containing short sequence tags or Expressed Sequence Tags (dbEST). Over 500,000 human cDNA clones have been
10 arrayed, and over 500,000 sequences are available in dbEST. Sequence clustering algorithms indicate that the majority of the 80,000 or so human genes are now likely to be represented by clones in this collection. Hillier, *supra*; Aaronson *et al.*, *Genome Research* 6:829-845 (1996).

15 The majority of clones available in the I.M.A.G.E. cDNA collection are from dT-primed mRNA, and the average length is between 1-2 kb. Therefore, the collection more fully represents the 3' 1-2 kb portion of many mRNAs but provides less coverage of 5' and coding sequences. Of the 4,200 human mRNAs collated in the human non-redundant mRNA set (NCBI), approximately 25% are fully represented by an I.M.A.G.E. clone, 50% have a partial representative, and less than 25% lack any representative clone. Based upon
20 resequencing of clones, the estimated overall error rate presently is about 9%. Thus, about 91% of the time, a clone thought to reside in a particular microtiter plate location is indeed the one grown up from that plate well.

25 It is specifically intended that these cDNA clones be used to provide the foreign genetic material according to the present invention. Moreover, it is fully contemplated that additional libraries from other biological sources and arrayed in differing manners may be suitably adapted to the present method without undue experimentation.

30 Despite the high yields and authenticity of processing associated with recombinant baculovirus expression systems, purification of the desired product from baculovirus infected cells is no easier than from any other eukaryotic system. Many expression vectors have been developed enabling synthesis of the antigen of interest as a fusion with a polypeptide facilitating purification. For instance, protein A fusion proteins may be affinity purified on IgG, polyarginine fusion proteins may be purified by cation exchange, polyhistidine fusions may be purified by virtue of their chelation of zinc ions, β -

galactosidase fusions, and other fusions to specifically immunogenic partners, may be purified by immunoaffinity, and β -galactosidase, maltose binding protein and glutathione-S-transferase (GST) fusion proteins may be purified by substrate affinity. Other epitope tags such as those recognized by the EE or Glu-Glu antipeptide antibody may also be used.

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15 recognized when placed at the C-terminus or at internal portions of the protein. The antibody is also useful for immunoprecipitates, immunofluorescence and western blots. Moreover, this antibody is produced at high levels by the EE hybridoma. Some have reported that 10 L preps routinely yield 3-5g of purified antibody. The EE tag has the additional advantage of containing a strong tyrosine phosphorylation substrate for protein
20 kinases such as src. Such a tag may be a useful assay detection label for high throughput protein interaction assays. Such a tag may be directly labeled with radioactive phosphate or detected with an anti-phosphotyrosine antibody.

In a fourth aspect, the present invention features a method for determining the function of foreign genetic material comprising the steps of: (a) providing a baculovirus transfer vector comprising one or more selectable marker genes, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites, (b) ligating foreign
25 genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter, (c) transfecting the baculovirus comprising the foreign genetic material into a host cell, and (d) observing the biological
30

results produced thereby. In preferred embodiments, the present invention is adapted to automation and thereby is useful for high throughput analysis.

Those of skill in the art will readily understand that there are many methods to determine the function of foreign genetic material once expression in a host, such as an insect cell is attained. In one embodiment the function of a nucleic acid may be determined by complementation analysis. That is, the function of the nucleic acid of interest may be determined by observing the endogenous gene or genes whose function is replaced or augmented by introducing the nucleic acid of interest. In a second embodiment, the function of a nucleic acid may be determined by analyzing the biochemical alterations in the accumulation of substrates or products from enzymatic reactions according to any one of the means known by those skilled in the art. In a third embodiment, the function of a nucleic acid may be determined by observing phenotypic changes in the host by methods including morphological, macroscopic or microscopic analysis. In a fourth embodiment, the function of a nucleic acid may be determined by observing the change in biochemical pathways which may be modified in the host as a result of the local and/or systemic expression of the foreign genetic material. In a fifth embodiment, the function of a nucleic acid may be determined utilizing techniques known by those skilled in the art to observe inhibition of gene expression in the cytoplasm of host cells as a result of expression of the foreign genetic material.

A particularly useful way to determine gene function is by observing the phenotype in an organism when a particular foreign genetic material has been expressed therein. Useful phenotypic traits in organisms which may be observed microscopically, macroscopically or by other methods include, but are not limited to, improved tolerance to insecticides, improved tolerance to extremes of heat or cold, drought, salinity or osmotic stress; improved resistance to diseases (fungal, bacterial or viral), production of enzymes or secondary metabolites; and the like. Other examples include the production of important proteins or other products for example antibodies, hormones, pharmaceuticals, antibiotics and the like. The phenotypic trait may also be a secondary metabolite whose production is desired.

One strategy that has been proposed to assist in such efforts is to create a database of expressed sequence tags (ESTs) that can be used to identify expressed genes. Accumulation and analysis of expressed sequence tags (ESTs) have become an important component of genome research. EST data may be used to identify gene products and

thereby accelerate gene cloning. Various sequence databases have been established in an effort to store and relate the tremendous amount of sequence information being generated by the ongoing sequencing efforts. It is specifically contemplated that the present method may be used to expedite efforts to isolate functional genes and determine the biological function thereof.

In a fifth aspect, the present invention features genes and fragments thereof, nucleotide sequences, and gene products obtained by way of the method of the present invention. The present invention features expressing selected foreign genetic material including nucleotide sequences in a host cell. Those of skill in the art will readily appreciate that the gene products of such foreign genetic material may be isolated using techniques known to those skilled in the art. Such gene products may exhibit biological activity such as pharmaceuticals and other similar functions.

The following definitions are provided to further clarify the present invention. No particular definition is intended to be limiting but is rather provided to clarify the meaning of the terms as used herein.

Expressed sequence tags (ESTs): Relatively short single-pass DNA sequences obtained from one or more ends of cDNA clones and RNA derived therefrom. They may be present in either the 5' or the 3' orientation. ESTs have been shown useful for identifying particular genes.

Expression: The term as used herein is meant to incorporate one or more of transcription, reverse transcription and translation.

Gene: A discrete nucleic acid sequence responsible for producing one or more cellular products and/or performing one or more intercellular or intracellular functions.

Host: A cell, tissue or organism capable of replicating foreign genetic material such as a nucleic acid and which is capable of being transfected with the same as, for example, by way of a baculovirus or transfer vector fragment thereof. This term is intended to include prokaryotic and eukaryotic cells, organs, tissues or organisms, where appropriate.

Transfer vector: A nucleotide sequence operable to transfer foreign genetic material into a host cell to effect subsequent transcription and/or translation of the foreign genetic material. Specifically intended within the scope of this term are baculoviruses and fragments thereof including those fragments which are linearized.

EXAMPLES OF THE PREFERRED EMBODIMENTS

The following examples further illustrate the present invention. These examples are intended merely to be illustrative of the present invention and are not to be construed as being limiting. The examples are intended specifically to illustrate that foreign genetic 5 material may be expressed by way of recombinant baculovirus expression systems in a host insect cell in an automated fashion to rapidly effect production of proteins and peptides and to subsequently assess the function thereof.

EXAMPLE 1

Selecting and re-arraying cDNA into 96-well format

We will choose a group of genes for which cDNA clones are available. We will re-array the cDNA clones into 96 plate format. Re-arraying robots such as those in use at Lawrence Livermore National Laboratories may be used for this project. We will sequence verify the identity of clones on an as needed basis. Some clones will be seeded into multiple wells to allow for preliminary estimates of reproducibility between wells. We will also 10 initiate the data tracking process such that information regarding clone identifiers, sequence accession numbers, passage number, etc are available for each re-arrayed plate. Clones chosen will also represent a mix of known and unknown clones. For the known clones, we 15 will include both some full and some partial representatives.

EXAMPLE 2

Amplification of arrayed cDNAs

We will directly transfect amplified cDNA that has been ligated to linear recombinant baculovirus expression systems (transfer vectors) containing the required recombination and expression elements. This will minimize the number of steps required to automate this process and provide maximum flexibility in handling a diverse collection of cDNA clones.

PCR primers appropriate for each cDNA library vector will be designed and synthesized. The primers will include restriction sites for directed ligation to the transfer vector fragments. cDNA inserts will be amplified using these primers, cut with the appropriate restriction enzymes, ligated to transfer vector fragments, purified if necessary and transfected to insect cells. The supernatant from these transfected cells will be passaged to 25 produce high-titer recombinant virus stock. The possibility of using undigested PCR products for directed single base overhang ligations will also be investigated. It is important to use the highest-fidelity thermostable polymerases available as well as the minimum 30 number of amplification cycles to reduce any sequence infidelities.

In order to assure that each cDNA insert is in proper reading frame with respect to the start codon in the transfer vector, the appropriate transfer vector for each cDNA clone must be selected. This may be done by programming a robotic pipettor to pick up the correct vector based on an analysis of the known 5' (EST) sequence of the cDNA clone.

5

EXAMPLE 3

Construction of Baculovirus transfer vectors to accept and adapt amplified cDNA

A new series of expression vectors will be constructed to facilitate the cloning of cDNA fragments from the cDNA libraries available. These expression vectors will take advantage of recent developments in insect cell expression. Recombinant baculoviruses can be generated by transfecting insect cells with ligated fragments containing transfer vector elements and cDNA inserts rather than cloned transfer vector plasmids. This greatly simplifies the process of obtaining recombinant baculoviruses by eliminating the plasmid cloning steps. These vectors will include unique restriction enzyme sites such as SfiI and NotI specific for the primers that will be used to amplify the cDNA inserts. The protein products expressed by these vectors will therefore have the EE tag at the N-terminus followed by 2-4 amino acids derived from a linker sequence.

A baculovirus apoptosis resistance gene (p35 gene) has been identified that confers increased viral yield in certain cell lines. Clem *et al.*, *Science* 254: 1388-1390 (1991).

Recombinant baculoviruses bearing this gene may be selectively amplified (up to 10^6 fold) in appropriate hosts. Lerch *et al.*, *Nucleic Acids Research* 21:1753-1760 (1993). We will include this gene in our vector design and eliminate it from our host virus strain to ensure that the percentage of recombinant baculovirus in our virus stocks is maintained at close to 100%. More specifically, the p35 apoptosis suppressor gene will be inserted upstream from the polyhedrin promoter. This will provide an additional selection for recombinant virus as the vectors will be used with a baculovirus strain that lacks the p35 gene. These vectors will be otherwise identical to the pAcoG and pAcOGS vectors from which they will be derived.

These vectors will be cotransfected with DNA from polyBsu2, a baculovirus strain developed at Cetus Corporation (Martha Stampfer and Robin Clark, unpublished). It contains three Bsu36I sites which, when cut, remove the essential ORF-1629 gene from the baculovirus genome. Thus the strain is very similar to a strain previously described. Kitts *et al.*, *Biotechniques* 14:810-817 (1993). The viability of Bsu36I digested polyBsu2 DNA can only be restored by recombination with transfer vector DNA containing ORF-1629. The

strain will be modified using standard recombination methods to inactivate the p35 apoptosis suppressor gene.

EXAMPLE 4

Preparing recombinant Baculovirus by direct transfection of ligated DNA

5 A number of aspects of insect cell culture may be optimized in order to develop particularly robust formats. For example, a Sf9 host cell inoculation may be optimized with regards to inoculation density for cells in 96 well plates.

In some experiments designed to simulate a high throughput and/or automated protocol, we used a Sf9 inoculum derived from a large culture, grew the inoculum in a serum 10 free insect medium, and cultured the cells in 96 well tissue culture plates at varying densities.

We achieved inoculum densities resulting in reproducible exponential growth over 24 hours. Thus, such cultures will allow the generation of cultures in optimal condition for both infection with recombinant baculoviruses and cotransfection with donor transfer vectors and acceptor baculovirus DNA.

15 EXAMPLE 5

We will optimize methods for producing proteins from recombinant baculovirus expression systems. However, it is presently contemplated that those methods presently available and described in the literature are acceptable. Miniaturization and automation of required operations including cell culture, infections, harvesting infected cells, preparing 20 lysates, affinity chromatography and storage of purified proteins may be optimized according to known procedures without undue experimentation.

EXAMPLE 6

Affinity purification of recombinant proteins using the Glu-Glu tag

Purifying the desired product from baculovirus infected cells may be performed according to any one of many protocols. Many expression vectors have been developed enabling synthesis of the antigen of interest as a fusion with a polypeptide facilitating purification. For instance, protein A fusion proteins may be affinity purified on IgG, polyarginine fusion proteins may be purified by cation exchange, polyhistidine fusions may be purified by virtue of their chelation of zinc ions, β -galactosidase fusions, and other fusions 30 to specifically immunogenic partners may be purified by immunoaffinity and β -galactosidase, maltose binding protein and glutathione-S-transferase (GST) fusion proteins may be purified by substrate affinity.

An epitope tag recognized by the EE or Glu-Glu antipeptide antibody may also be used. Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci.* 82: 7952-7954 (1985). This antibody was raised against a peptide that comprises the major tyrosine phosphorylation site of polyoma middle T antigen. Talmage *et al.*, *Cell* 59:55-65 (1989) The use of this tag for protein purification was developed at Cetus Corporation (Rubinfeld *et al.*, *Cell* 65:1033-1042 (1991)) and has been the primary tag used for this purpose at Chiron Corporation. The epitope for this tag has been fully characterized and optimized by Mimotope peptide scan analysis (Mario Geysen, John Wang and Robin Clark, unpublished data). The antibody has moderate affinity for the tag (K_d approximately 2×10^{-7}) which allows rapid elution of tagged proteins by free peptide under non-denaturing conditions while retaining efficient binding of proteins in crude lysates. While usually placed at the N-terminus, the tag is recognized when placed at the C-terminus or internal portions of the protein. The antibody is also useful for immunoprecipitates, immunofluorescence and western blots. Garcia *et al.*, *Mol. Cell Biol.* 13:6615-6620 (1993) Supply of this antibody is not a problem as it is produced by the EE hybridoma at high levels. 10 L preparations have routinely yielded 3-5 g of purified antibody. The EE tag has the additional advantage of containing a very strong tyrosine phosphorylation substrate for protein kinases such as src. Garcia *et al.*, *J. Biol. Chem.* 268:25146-25151 (1993) This feature will be useful as an assay detection label for high throughput protein interaction assays. It may be directly labeled with radioactive phosphate or detected with an anti-phosphotyrosine antibody.

The use of the EE tag has been facilitated by a series of baculovirus expression vectors, designated as pAcOG1, 2, and 3, in which the original polylinker of pAcC13 was replaced with a synthetic polylinker encoding an initiating methionine, the Glu-Glu epitope tag and multiple single restriction enzyme sites in all three frames. Munemitsu *et al.*, *Mol. Cell. Biol.* 10:597-5982 (1990). These vectors allow one-step construction of baculovirus expression vectors in which insert coding sequence (with native stop codon) is fused to an N-terminal EE tag in frame. Another set of vectors, pAcO G1S, 2S. and 3S, also contain a series of tandem stops in all three frames for partial constructs which do not bring along their own stop codon. The order of the sites was influenced by the most commonly found restriction enzymes utilized for the creation of cDNA libraries. The vector itself was optimized for expression by including unique sequences upstream and downstream of the initiating methionine which were experimentally determined to enhance expression in Sf9 cells. These vectors have been used to produce many EE-tagged proteins.

To demonstrate that Glu-tagged proteins may be conveniently immunoassayed in a 96-well format amenable to high throughput automation, we performed the following experiment. Millipore MAIP N45 high protein binding plates containing Immobilon-P filtration membranes were treated with either Glu-tagged or GST-tagged proteins and assayed 5 for binding using a monoclonal anti-Glu-Glu antibody and an alkaline phosphatase conjugated goat anti-mouse IgG secondary antibody. Alkaline phosphatase was detected using SigmaFast BCIP/NBT tablets. As shown in Figure 4, purified Rho-EE, E2F-EE and Myt1-EE were detected at concentrations ranging from 0.1 to 2.0 µg/well. These results demonstrate that the Glu-tag is a convenient and specific immunoassay target for quantifying 10 recombinant proteins in a 96-well format. Furthermore, this technique is easily adapted to a limiting dilution assay for screening of recombinant baculovirus, and could also be adapted for *in situ* detection of recombinant proteins in a 96-well tissue culture wherein they have been produced.

EXAMPLE 7

15 These data demonstrate that it is feasible to perform an enzyme assay on a baculovirus-expressed recombinant protein in the same 96-well format in which the Sf9 cells were seeded and infected with virus. We used a virus expressing an enzyme which can easily be assayed colorimetrically (e.g. Gus). However, the same principles may be extended to proteins with other functions.

20 Enzymatic detection of recombinant proteins in a 96-well plate format was tested using a recombinant baculovirus expressing β-glucuronidase (Gus). Sf9 cells grown in insect serum free medium were grown to mid-log phase and used to seed 96-well flat bottom polystyrene tissue culture plates at a density of 40,000 cells/100µl/well. rGus virus was serially diluted in 10-fold increments. After addition of virus dilutions, the plate was 25 incubated for several days, and then X-glucuronide stock was added to each well. X-glucuronide is a histochemical substrate for β-glucuronidase, yielding a blue substrate upon hydrolysis.

Blue color development was visible in many wells within minutes after addition of 30 the substrate, and color development continued to occur in high dilution wells over 24 hours as rGus production increased in infections resulting from low MOI's. Color development was visible both in the supernatant of infected wells as well as within individual cells when examined microscopically. These results as demonstrated in Figure 3 indicate that enzymatic activity is easily detectable in baculovirus infected cultures where

low cell numbers are infected at low MOI. Thus, this and related enzymatic assays *in situ* following baculovirus infection may be adaptable to high throughput automation.

EXAMPLE 8

This example demonstrates some principles of the present invention wherein a recombinant baculovirus is produced from a cDNA clone in a miniaturized (96-well) format with no cloning steps. The principle elements of this procedure are diagrammed in Figure 2, discussed *supra*.

Step 1: Bacterial cultures containing a cDNA clone plasmid are arrayed in a 96-well plate. The plasmid is a Bluescript cDNA library vector which contains the GUS gene, which is commonly used as an expression marker.

Step 2: The GUS gene insert is amplified by PCR procedures using primers that hybridize to a plasmid vector sequence on either side of the cDNA insertion site. These primers also contain restriction sites (Not1 for the 5' primer and Sfi1 for the 3' primer) in the 5' ends of the primers. The portion of the primer containing the restriction sites does not hybridize to the plasmid vector sequence, but is incorporated into the PCR product.

Step 3: The PCR products from step 2 are digested with Not1 and Sfi1 restriction endonucleases to generate the appropriate sticky ends on the products.

Step 4: The restriction fragments generated in step 3 are purified by chromatography on an Arrayit 96-well DNA purification apparatus.

Step 5: Baculovirus transfer vector arms are generated by digesting pAcOP2 with Not1, Sfi1, Pvu2 and Xho1. The pAcOP2 plasmid contains the EE epitope tag followed by a cloning site containing Not1 and Sfi1 restriction sites. The EE tag includes a start codon and is positioned to be transcribed by a polyhedrin promoter. pAcOP2 also contains the orf-629 and p35 selectable markers and thus is an example of the baculovirus transfer vector depicted in Figure 2, described *supra*. The DNA from this restriction digest is purified by chromatography on a Qiagen column.

Step 6: The restriction fragments from steps 4 and 5 are mixed and ligated together with DNA ligase.

Step 7: Baculovirus DNA containing a BsU36I restriction endonuclease site in the orf-1629 region (poly BsU2) is digested with BsU36I to generate linearized fragments that lack a complete orf-1629 gene.

Step 8: The ligation products from step 6 are co-transfected into insect (Sf9) cells (seeded in a 96-well plate) with baculovirus DNA from step 7 using standard insect cell transfection protocols such as the Invitrogen lipofection method.

5 Step 9: The transfected Sf9 cultures from step 8 are incubated 5 days. The resulting baculovirus stock is passaged 2 times on Sf9 cells.

Step 10: The passaged virus stock from step 9 is diluted 10-fold into medium containing 150 ug/ml X-gluc and used to infect Sf9 cells in a 96-well plate. Eight of the 96 wells are used for experimental controls.

10 Step 11: Two days after infection the plate from step 10 is scored for the presence of a blue color in the wells, which indicates the expression of the GUS gene. The percentage of blue wells relative to the total number of wells indicates the efficiency and degree of success of the process.

WHAT IS CLAIMED:

1. A baculovirus expression system comprising at least one selectable marker gene, a promoter, a transcription termination and a cloning site operable to accept foreign
5 genetic material.
2. The baculovirus expression system of claim 1 wherein the promoter comprises a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof.
3. The baculovirus expression system of claim 1 wherein the transcription termination sequence comprises a transcription termination sequence of a polyhedrin structural
10 gene or fragment thereof.
4. The baculovirus expression system of claim 1 further comprising a tag and one or more restriction sites.
5. The baculovirus expression system of claim 1 wherein the selectable marker gene is the apoptosis suppressor p35 gene.
- 15 6. The baculovirus expression system of claim 5 wherein said apoptosis suppressor gene is located upstream from the polyhedrin promoter.
7. A method for producing a recombinant baculovirus expression system comprising the steps of
 - (a) providing a baculovirus transfer vector comprising at least one selectable marker gene, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites; and
20
 - (b) ligating foreign genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter.
25
8. The method of claim 7 wherein said baculovirus transfer vector is linearized.

9. The method of claim 7 wherein said foreign genetic material is cDNA.
10. The method of claim 7 wherein said ligating is performed by automation in an array of multiple wells.
11. A method for expressing foreign genetic material comprising the steps of:
 - 5 (a) providing a baculovirus transfer vector comprising an apoptosis suppressor gene, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites;
 - 10 (b) ligating foreign genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter; and
 - (c) transfecting the baculovirus transfer vector comprising the foreign genetic material into a host cell.
- 15 12. The method of claim 11 further comprising the step of purifying the gene product produced thereby.
13. The method of claim 12 wherein said purifying is performed by immunoassay of a tag epitope.
- 20 14. The method of claim 11 wherein said baculovirus transfer vector is linearized.
15. The method of claim 11 wherein said foreign genetic material is cDNA.
16. The method of claim 11 wherein said ligating is performed by automation in an array of multiple wells.
17. The method of claim 11 wherein the host cell comprises a baculovirus genome.
- 25 18. The method of claim 11 wherein the host cell comprises a baculovirus genome with the proviso that the baculovirus genome does not comprise an apoptosis suppressor gene.

19. A method for determining the function of foreign genetic material comprising the steps of:

- (a) providing a baculovirus transfer vector comprising an apoptosis suppressor gene, a promoter such as a polyhedrin promoter of a polyhedrin structural gene or a fragment thereof, a transcription termination sequence such as a transcription termination sequence of a polyhedrin structural gene or fragment thereof, a cloning site operable to accept foreign genetic material, optionally a tag and optionally one or more restriction sites;
- (b) ligating foreign genetic material into the baculovirus transfer vector at the cloning site such that the foreign genetic material is under the control of the promoter;
- (c) transfecting the baculovirus transfer vector comprising the foreign genetic material into a host cell; and
- (d) observing the biological results produced thereby.

20. The method of claim 19 wherein said observing comprises analyzing biochemical alterations in the accumulation of substrates or products from enzymatic reactions.

21. The method of claim 19 wherein said observing comprises comparing an RNA expression profile in a first cell comprising the foreign genetic material with an RNA expression profile in a second cell with the proviso that the second cell does not comprise the foreign genetic material of interest.

22. A product selected from the group consisting of a gene or a fragment thereof, a nucleotide sequence, and a gene product produced according to the method of claim 11.

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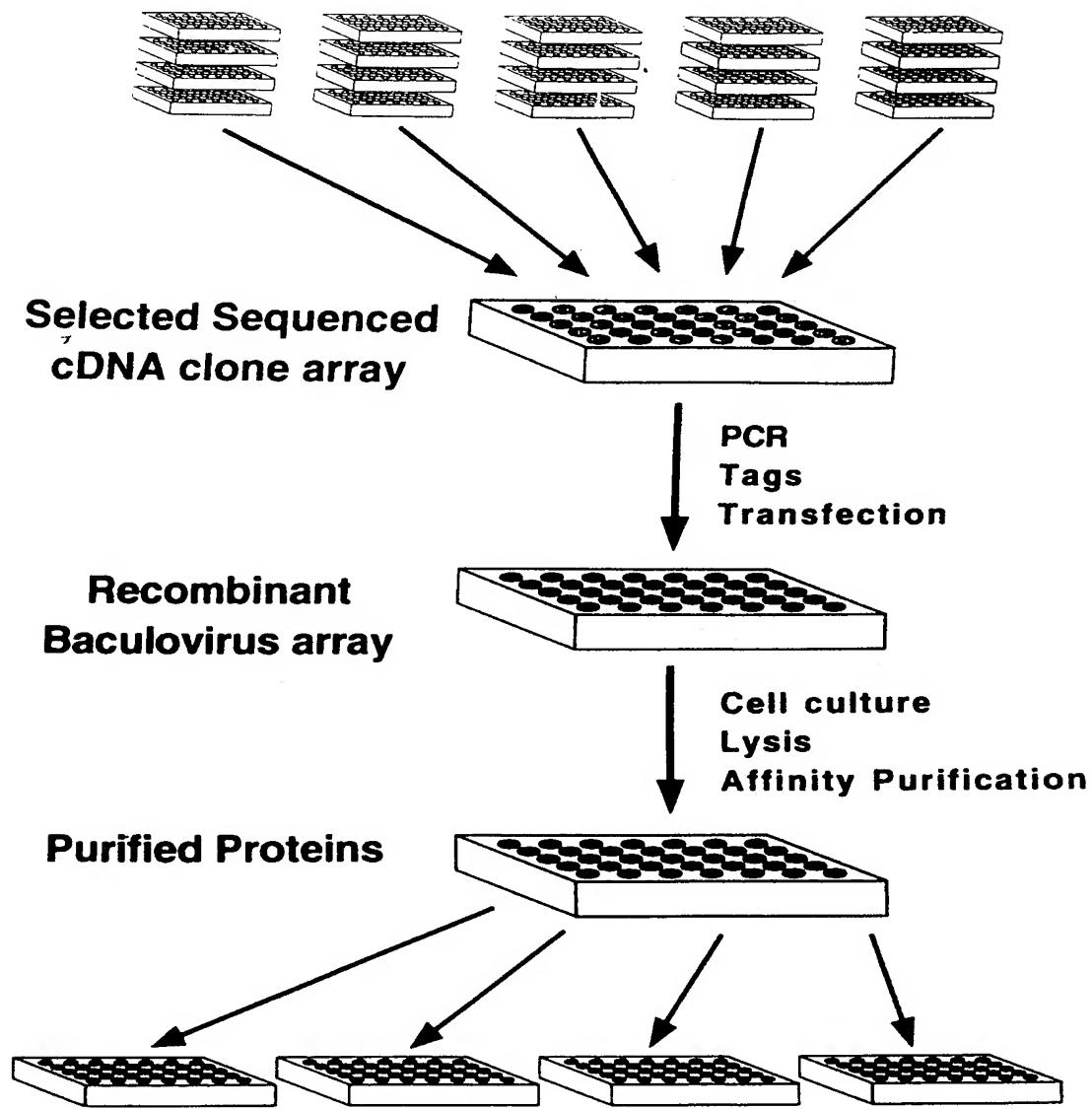


FIGURE 1

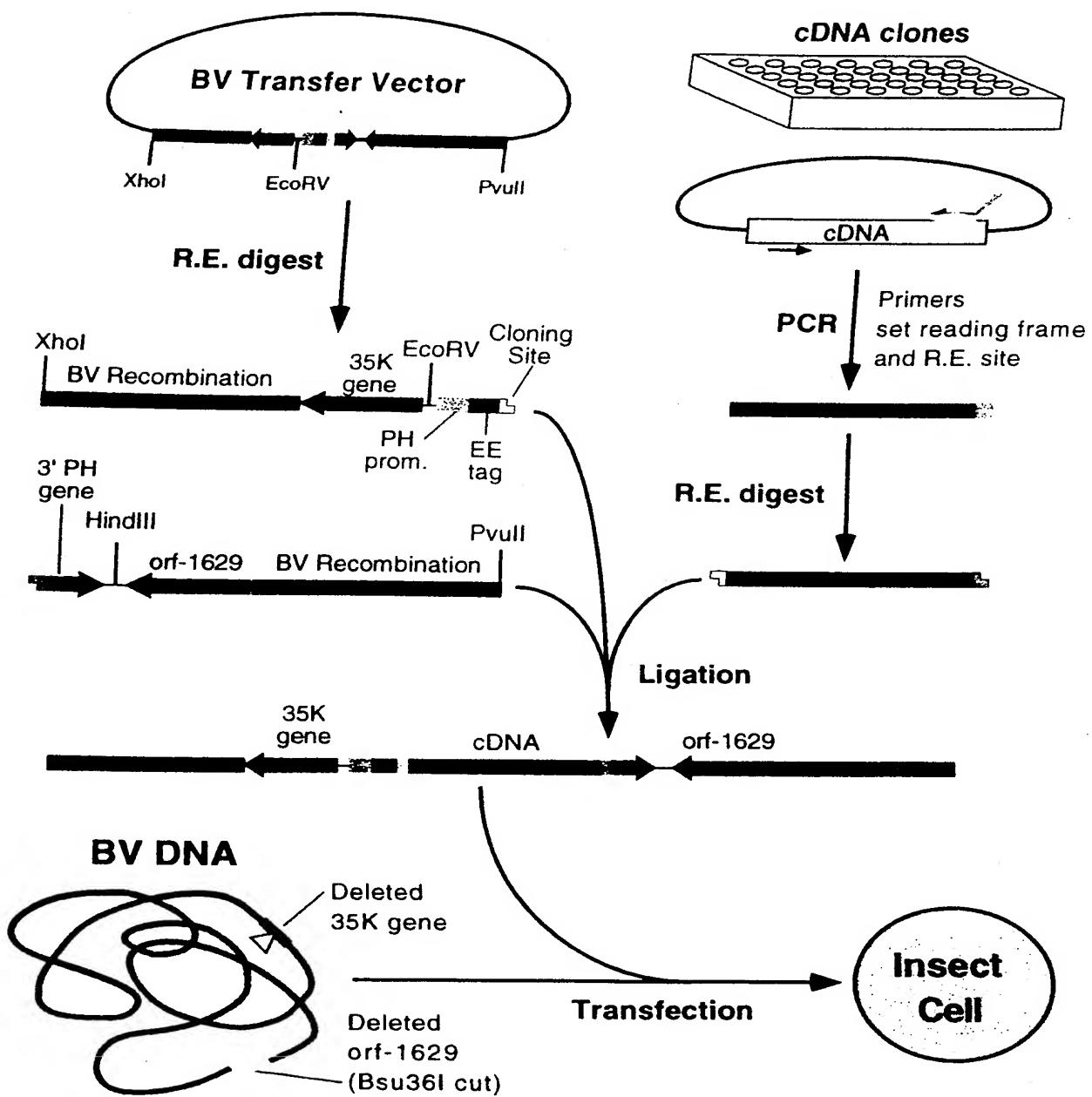


FIGURE 2

3/5

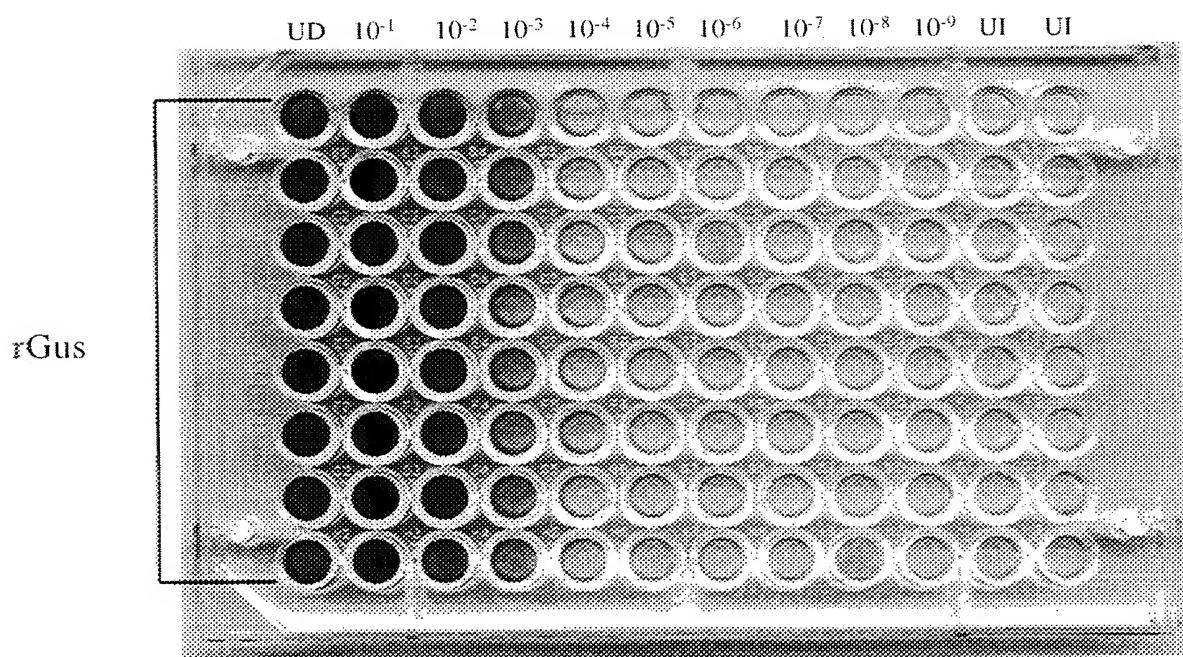


FIGURE 3

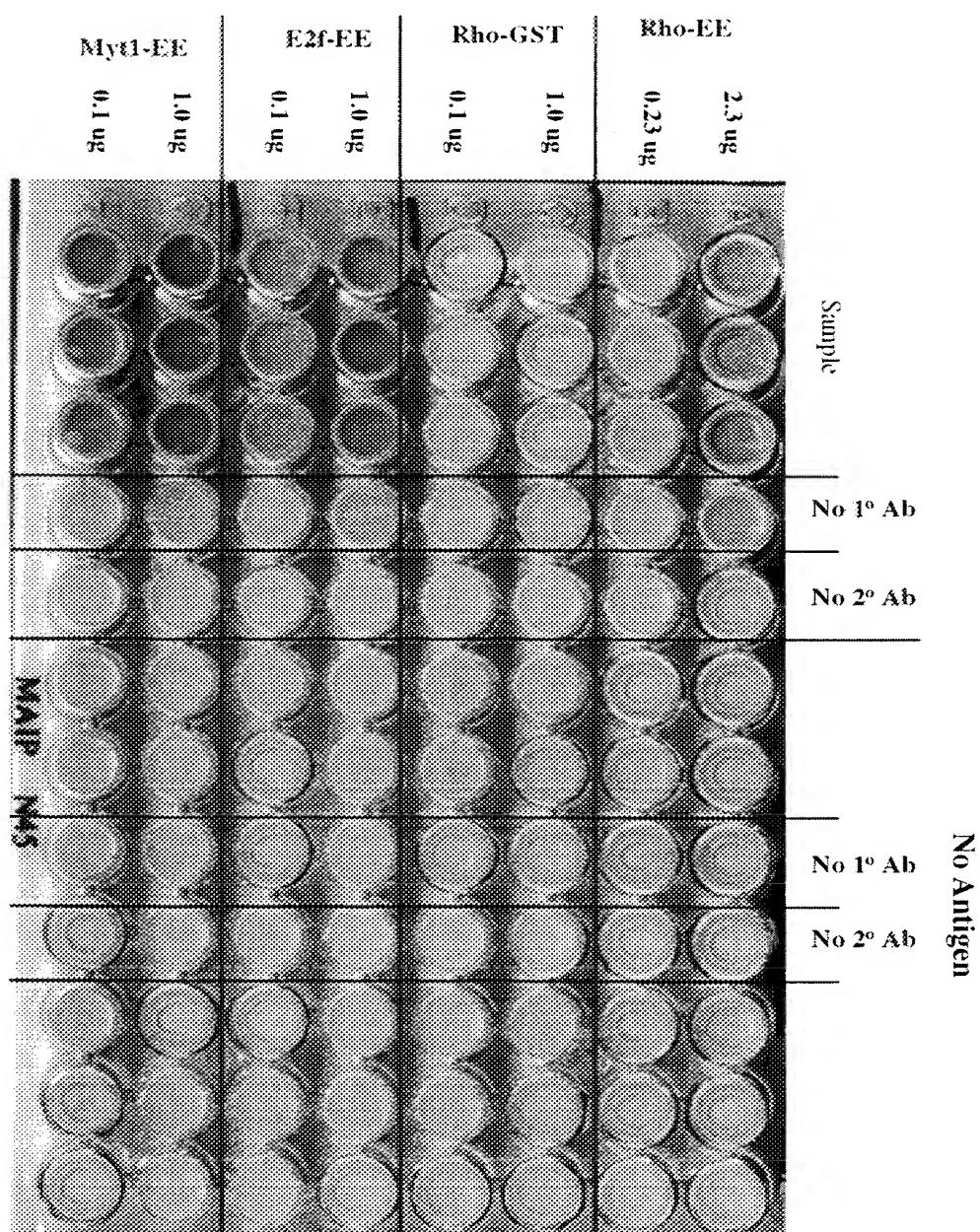


FIGURE 4

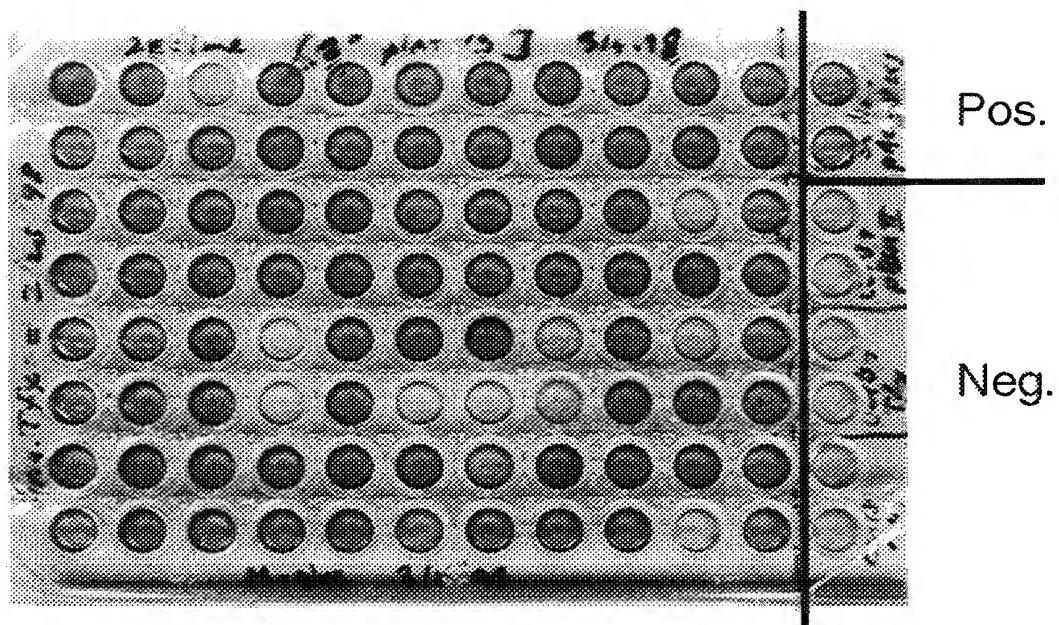


FIGURE 5

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/04918

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LUCKOW, V.A.: "Baculovirus systems for the expression of human gene products" CURRENT OPINION IN BIOTECHNOLOGY, vol. 4-5, - 1993 pages 564-572, XP002109733 see page 566, right-hand column, lines 19-25	1-3, 5, 6
A	see page 565, "New baculovirus transfer vectors"; page 566, "Use of selectable and counter-selectable markers" ---- -/-	1

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

20 July 1999

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INTERNATIONAL SEARCH REPORT

national Application No

PCT/US 99/04918

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LERCH, R.A. AND FRIESEN, P.D.: "The 35-kilodalton protein gene (p35) of <i>Autographa californica</i> nuclear polyhedrosis virus and the neomycin resistance gene provide dominant selection of recombinant baculoviruses" NUCLEIC ACIDS RESEARCH, vol. 21, no. 8, 1993, pages 1753-Nu1760 , XP002091606 cited in the application see page 1754, "Transfer plasmids" see the whole document	1-3,5,6
A	---	1
X	COLEMAN, T.A. ET AL.: "Production and purification of novel secreted human proteins" GENE, vol. 190, no. 1, 29 April 1997 (1997-04-29), pages 163-171, XP004064398 see page 164, section 2.1 see page 164, section 2.1	1,7
A	---	1
X	PAIN, M.J.I. ET AL.: "Functional high-level expression of cytochrome-P450 CYP2D6 using baculoviral expression systems" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 328, no. 1, 1 April 1996 (1996-04-01). pages 143-150. XP002109734 see page 145, "CYP2D6 expression" see pages 114-145, "Baculovirus constructs" and "Plate assay"; pages 147-148, "Plate assay"	22
A	---	1
A	RUBINFELD, B. ET AL.: "Molecular cloning of a GTPase activating protein specific for the Krev-1 protein p21rap1" CELL, vol. 65, 14 June 1991 (1991-06-14), pages 1033-1042, XP002109735 cited in the application see page 1040, right-hand column, lines 7-9	1

	-/-	

INTERNATIONAL SEARCH REPORT

national Application No

PCT/US 99/04918

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KITTS, P.A. AND POSSEE, R.D. : "A method for producing recombinant baculovirus expression vectors at high frequency" BIOTECHNIQUES, vol. 14, 1993, pages 810-817, XP002100322 cited in the application see the whole document ----	1
A	DAVIES, A.H.: "Current methods for manipulating baculoviruses" BIO/TECHNOLOGY, vol. 12, no. 1, 1994, pages 47-50, XP002109736 see the whole document ----	1
A	KITTS, P.A.: "Construction of baculovirus recombinants" CYTOTECHNOLOGY, vol. 20, no. 13, 1996, pages 111-123, XP002109737 see the whole document -----	1